

Exhibit 1 - Kipps et al., J. Exp. Med., 161, 1-17, 1985

IMPORTANCE OF IMMUNOGLOBULIN ISOTYPE IN HUMAN ANTIBODY-DEPENDENT, CELL-MEDIATED CYTOTOXICITY DIRECTED BY MURINE MONOCLONAL ANTIBODIES

BY THOMAS J. KIPPS,* PETER PARHAM,² JENNIFER PUNT,* AND
LEONARD A. HERZENBERG*

*From the Departments of *Genetics and ²Cell Biology, Stanford University School of Medicine,
Stanford, California 94305*

There is considerable interest in using monoclonal antibodies (mAb)¹ as therapeutic adjuncts in treating human malignancy and in tissue transplantation (1, 2). Several clinical trials involving tumor-specific murine mAb in the treatment of lymphomas (3, 4) have yielded promising results. However, the response to mAb in several other studies has been less satisfactory (5-7), indicating the presence of unknown variables that may strongly influence the outcome of such therapy.

Critical to our appreciation of these variables is a better understanding of the interaction(s) between mouse Ig and the effector cells of the human immune system. mAb are suspected of mediating tumor cytotoxicity, not by activating complement (C), but by directing antibody-dependent, cell-mediated cytotoxicity (ADCC) (8). Indeed, tumor-specific Ig administered to mice depleted of C by cobra venom, retards the growth of transplanted tumor as readily as Ig administered to animals with normal levels of C3 (9). Thus, the capacity of Fc receptor (FcR)-bearing killer (K) cells to recognize the heavy chain (H) of mouse Ig may be an important factor in the response to mAb therapy.

Important in this regard is whether the H isotype of a murine mAb influences its capacity to direct ADCC by human effector cells. While some investigations (10, 11) indicate that all mouse mAb isotypes may direct ADCC by human effector cells, Koprowski and colleagues argue that only IgG2a is effective, at least in directing ADCC by human monocytes and macrophages (12). This latter contention is supported by the observation that cultured human monocytes interact only with murine IgG2a in ADCC (13). Moreover, Hellstrom et al. (14) and Imai et al. (15) have data suggesting that murine IgG2a antitumor antibodies are more efficient than IgG1 antibodies of like specificity in directing ADCC mediated by human K cells. Quantitative comparison of different mouse Ig

This work was supported by grants GM17367, CA 04681, and AI01897. T. J. Kipps is a special fellow of the Leukemia Society of America.

¹Abbreviations used in this paper: ADCC, antibody-dependent, cell-mediated cytotoxicity; DNP, dinitrophenyl; E/T, effector/target; FACS, fluorescence-activated cell sorter; FcR, Fc receptor; FITC, fluorescein isothiocyanate; H chain, heavy chain; K, killer; mAb, monoclonal antibody; PBL, peripheral blood lymphocyte; RIA, radioimmunoassay.

J. Exp. Med. © The Rockefeller University Press · 0022-1007/85/01/0001/17 \$1.00
Volume 161 January 1985 1-17

isotypes for their ability to direct ADCC, however, has not been possible, because each of the mAb tested has been an independent hybridoma product with unique target antigen fine specificity and affinity. Precise analysis of the role of isotype in mediating ADCC would require mAb of different isotypes with identical binding sites, to exclude the possibility that differences in the binding specificities or affinities are responsible for differences in ADCC.

mAb fulfilling this requirement can be generated by selecting switch variant hybridoma cells which spontaneously express an H isotype different from that of the parent hybridomas, but retain expression of the same H variable region and light chains (16-18). Ig produced by members of such switch variant families have identical reactivities with antigens and differ only in isotype, thus permitting study of the dependence of isotype in ADCC with no other antibody variables. Our present studies on ADCC mediated by human peripheral blood lymphocytes (PBL) are directed by IgG1, IgG2b, or IgG2a produced by members of two switch variant families specific for two HLA antigens expressed by cells of a human B lymphoblastoid line, JY.

Materials and Methods

Cells. The human B lymphoblastoid cell line, JY, was used as the target cell in the ADCC assay. This cell expresses HLA antigens A2, B7, and DC-1 (19). ME1 and MA2.1 are murine hybridoma cell lines. ME1, developed by Ellis et al. (20), secretes an IgG1 with high affinity to HLA-B7, Bw22, Bw42, and B27. In addition to these strong reactions, this antibody cross-reacts weakly with HLA-B14 and a few other HLA-B locus products. MA2.1 secretes an IgG1 with high affinity for HLA-A2 and B17 (21). We selected the IgG2a and IgG2b isotype switch variants of ME1 and MA2.1 from their respective parent IgG1-producing hybridomas using the fluorescence-activated cell sorter (FACS), as described below. Cells were cultured in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 15% fetal calf serum (FCS) and 2 mM glutamine, and maintained at 37°C in a humidified atmosphere with 7% CO₂ in air.

Monoclonal Antibodies. mAb specific for Leu-2a, Leu-7, Leu-11a, and Leu-15 were gifts from the Becton Dickinson Monoclonal Center, Inc. (Mountain View, CA). Mo2 was the gift of Coulter Immunology (Hialeah, FL). The IgG1 anti-DNP (dinitrophenyl) mAb and Texas Red-avidin were the gifts of Dr. R. Hardy (Genetics Dept., Stanford University, Palo Alto, CA). Antibodies 8.3, reactive with Igh-1a, and 21-48.3, reactive with Igh-1a, 3a, are as described (22).

Fluochrome Conjugation of Antibodies. Isotype-specific goat anti-mouse antibodies and mAb 8.3 were conjugated to fluorescein isothiocyanate (FITC) as described by Goding (23).

Multiparameter FACS Analysis. Immunofluorescence and light scatter analysis of PBL were performed on a dual laser FACS II as described (24). Fluorescein and phycoerythrin were excited at 488 nm by an argon laser. Texas Red was excited by an adjustable rhodamine dye laser at 600 nm.

ADCC Assay. 80- μ l aliquots containing 5×10^5 ⁵¹Cr-labeled JY target cells in RPMI 1640 culture medium with 10% heat-inactivated FCS were added to individual wells of a 96-well V-bottom microtiter plate. Purified antibody (from which aggregates were removed by centrifugation in a Beckman airfuge for 15 min at 30 psi) was added to each well in 20 μ l isotonic phosphate-buffered saline (PBS), pH 7.4 to the final concentrations specified in the text. We incubated the cells with antibody for 20 min at room temperature, before adding 100 μ l of effector cells in culture medium to each well, at the specified effector/target (E/T) ratio. The plates were spun for 5 min at 200 g, and subsequently placed in a 37°C incubator with humidified 7% CO₂ in air. At times indicated in the text, the plates were again spun for 5 min at 200 g and 100 μ l of supernatant were harvested from each well. Samples were counted in a Beckman gamma scintillation counter. Wells

of labeled JY with neither antibody or effector cells served as spontaneous release controls. Control wells of labeled JY with either antibody or effector cells were included in each experiment. Maximum counts were from wells of labeled JY to which we added 100 μ l of 5% Triton-X (Sigma Chemical Co., St. Louis, MO) instead of effector cells. The percent specific release was determined by the formula: $[(\text{sample} - \text{spontaneous release}) / (\text{maximum counts} - \text{spontaneous release})] \times 100$. All samples were done in triplicate. Standard deviation of the mean for a given sample was calculated using an unbiased estimator for small samples (25).

Isolation of Effector Cells for ADCC. Blood samples from healthy volunteers were gathered in heparinized syringes and diluted 1:1 with RPMI 1640 at room temperature. Leukocytes were isolated on Ficoll-Hypaque (Pharmacia, Inc., Uppsala, Sweden) density gradients ($\rho = 1.08$ g/ml). Mononuclear cells were harvested and washed three times before assay. Cell viability, as assessed by staining with acridine orange and ethidium bromide, was >95%. Where indicated, adherent cells were removed by incubating the isolated PBL on plastic culture dishes (Falcon Labware, Oxnard, CA) for 2 h at 37°C in culture medium supplemented with 10% heat-inactivated FCS.

Radiiodination of Antibodies. Purified anti-IgH 1a (8.3) or goat anti-mouse IgG mAb was radiolabeled with ^{125}I using the method of Hunter and Greenwood (26).

Solid Phase Radioimmunoassay (RIA). mAb 21-48.3, anti-IgH 1a,3a (22), was used to coat polystyrene microtiter plates as described (27). After incubation with varying dilutions of culture supernates, and subsequent washing to remove unbound antibody, radiolabeled 8.3 (anti-IgH 1a) or goat anti-IgG2b was used to detect IgG2a or IgG2b, respectively.

Preparation of Ig and Antibody Fragments. Antibodies were isolated from the ascites of hybridoma-bearing mice by ammonium sulfate salt precipitation and subsequent DEAE, then Sephadex G-200 size chromatography. The IgG2a and IgG2b MA2.1 were purified using Staph A-Sepharose in lieu of G-200 Sephadex chromatography as described (28). The Fab' and the Fab'2 of the IgG1 ME1 were prepared as described (29). Goat antibodies specific for either mouse IgG1 or IgG2 were prepared as reported previously (30).

Switch Variant Selection. IgG1-producing parent hybridoma cell lines MA2.1 and ME1 had been growing in tissue culture a minimum of 4 mo without subcloning. Since the proportion of switch variants within a given hybridoma population will generally increase with time (31), these lines were not subcloned before selection. Switch variant selection with fluorescein-conjugated antibodies specific for mouse IgG2 (goat anti-mouse IgG2) or IgG2a (mAb 8.3) was performed on a modified FACS II (B-D FACS Systems, Sunnyvale, CA) using a cloning apparatus that permits pauci-population sorting as described (18, 31). We took care that cell populations used for switch variant selection were >95% viable before staining. In addition, propidium iodide was used to stain dead cells so they could be excluded from FACS analysis and sorting (17). Pauci-population sorting permitted the cloning of switch variants after one round of selection. Culture supernatants of wells into which 1, 5, 25, or 100 brightly fluorescent cells had been sorted were screened using a solid phase RIA for detection of the variant isotype. Cells that scored positive for the variant isotype were then stained again with FITC-goat anti-mouse IgG2 or FITC-8.3 (anti-IgH 1a). Single, stained cells were deposited into individual wells of microtiter plates. The proportion of IgG2b or IgG2a variants in either the ME1 or MA2.1 parent hybridoma populations was calculated by multiplying the initial sort frequency by the percentage of cells identified as being switch variants in the sorted plates, using the Poisson distribution to calculate the number of variants per plate from data obtained in the RIA (18, 31).

Cell-binding Assays. Radio-immune cell-binding assays to quantitate bound antibody were performed as described (32). Fluorescence-immune cell-binding assays to quantitate mouse antibody bound to JY were performed using FITC-conjugated goat anti-mouse Ig to stain washed cells that had been incubated for 20 min with anti-HLA at specified concentrations. Propidium iodide at 1 μ g/ml was used to stain dead cells so they could be excluded electronically from FACS analysis. Fluorescence data were collected using a modified FACS II equipped with logarithmic amplifiers. The mean fluorescence of 10^4 cells from each sample was subsequently converted to linear units for comparison of

fluorescence intensity between samples. The percent of maximal binding was calculated by dividing this value by the maximum mean fluorescence value achieved with a saturating concentration of tested antibody.

Results

Isolation of Switch Variant Families. The IgG1-producing hybridomas ME1 and MA2.1 were initially stained with isotype-specific, FITC-labeled, goat anti-mouse antibodies and found to be 90 and 97% positive for surface IgG1 expression, respectively. Except for an extremely small fraction of cells in either population, no appreciable staining with goat anti-mouse IgG2 antibody was detected. The proportion of IgG2b switch variants within the ME1 hybridoma population, however, was high enough to permit direct cloning during the first round of variant selection (Table I). The IgG2a ME1, and IgG2b and IgG2a MA2.1 switch variants were cloned from sorted pauci-populations found to producing switch variant antibody by RIA. >95% of the cells from each cloned switch variant line stained positively for the newly acquired variant isotype using isotype-specific, FITC-conjugated antibody. Furthermore, <1 per 10^5 cells from such cloned switch variant lines stained for the parent surface IgG1.

Quantitative Binding of Purified Antibodies Produced by the Switch Variant Families. Purified IgG1, IgG2a, and IgG2b of the ME1 switch variant family were compared by a radio-immune cell-binding assay to verify that no changes in either the specificity or avidity of the antibodies accompanied the isotype switch. As seen in Fig. 1a, titration of all three isotypes against a fixed number of JY cells in an indirect trace-binding assay gave superimposable binding curves. The same assay, using a single saturating concentration of IgG (25 μ g/ml), demonstrated that the binding of each ME1 isotype, to a panel of 12 B lymphoblastoid cell lines of different HLA types, was the same (data not shown). Similarly, titration of the MA2.1 switch variant family of antibodies, in a fluorescence-immune cell-binding assay, demonstrated that all three have identical binding activities for JY (Fig. 1b). These results confirm that the switch in the H constant region from IgG1 to IgG2a or IgG2b has no discernible effect on the antigen-combining site of these anti-HLA antibodies. Thus, the Ig produced by these

TABLE I
Number of Isotype Switch Variants Detected in Hybridoma Populations

Hybridoma cell line	Average number of variants ($\times 10^{-4}$)	
	IgG2b	IgG2a
ME1	1,600	64
MA2.1	32	8

Selection of switch variant families. Brightly fluorescent cells from 3×10^6 hybridomas of either cell line stained with FITC-goat anti-mouse IgG2 were sorted into individual wells of microtiter plates using the technique of pauci-population sorting. Wells containing variants of either subclass were subsequently identified using a solid phase RIA. The average number of variants per sorted plate was calculated using the Poisson distribution.

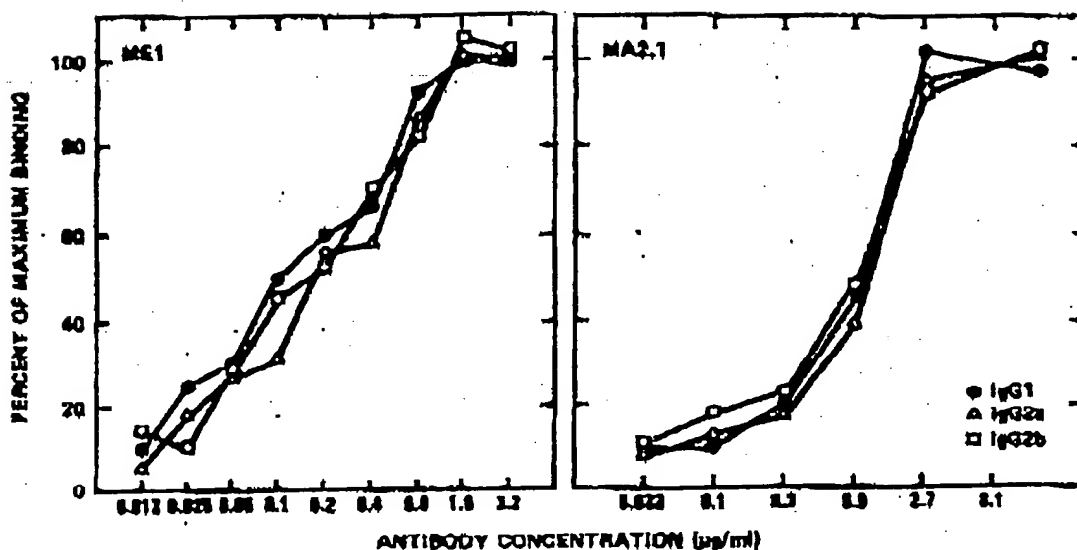


FIGURE 1. Titration of antibodies from switch variant families with JY: IgG1, IgG2a, and IgG2b antibodies of the ME1 switch variant family (left), or the MA2.1 family (right), were titrated with a fixed number of JY. Specifically bound antibody was detected with 125 I-labeled goat anti-mouse Ig (left), or with FITC-labeled goat anti-mouse Ig (right), for the radio-immune, or fluorescence-immune cell-binding assays, respectively. IgG1 (●), IgG2a (Δ), IgG2b (□).

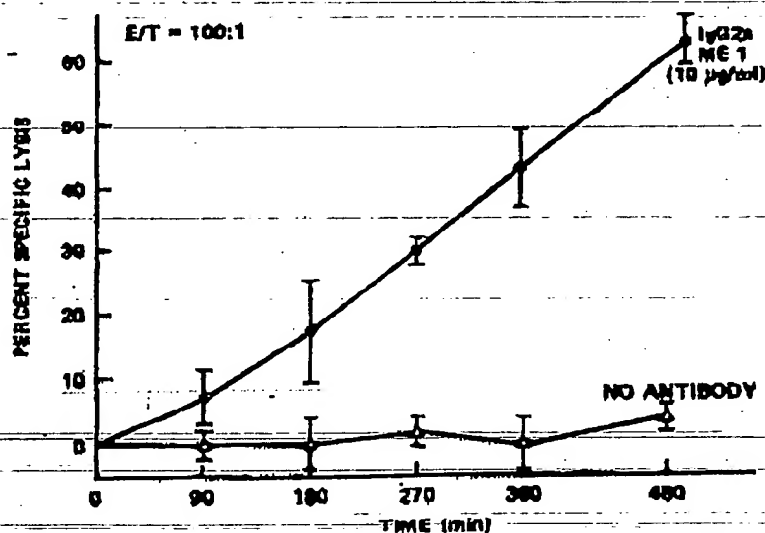


FIGURE 2. ADCC of IgG2a ME1 antibody: 51 Cr-labeled JY was incubated with or without IgG2a ME1 at 10 μ g/ml, as described in Materials and Methods. PBL were added to an E/T ratio of 100:1. Bars indicate standard deviation (\pm SD) of percent specific lysis for triplicate samples.

switch variant families were judged suitable for examining the effects of isotype on ADCC.

Antibody-dependent Cell Cytotoxicity. Our preliminary studies demonstrated that the IgG2a ME1 can direct significant ADCC of JY by freshly isolated human PBL. Specific lysis of antibody-coated JY increased linearly with time, reaching a maximum of ~80% lysis in the 6 h period (Fig. 2). No significant lysis occurred in the absence of antibody, nor with nonspecific IgG2a antibody. Furthermore,

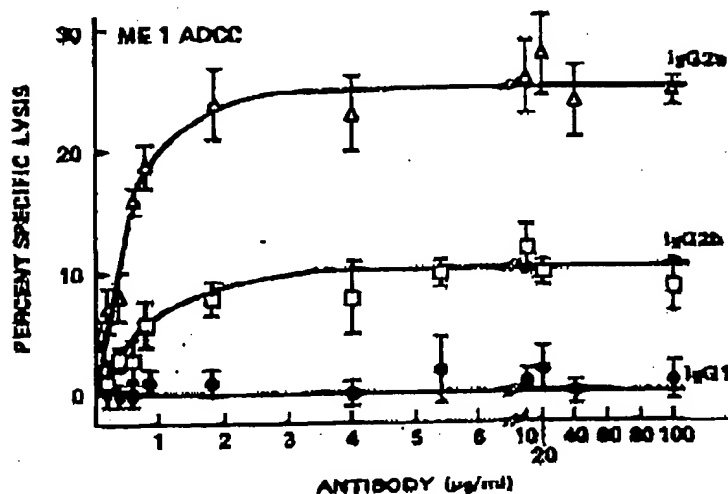


FIGURE 3. Comparison of ADCC activities of different antibody isotypes of ME1. Antibody at various concentrations was added to ^{51}Cr -labeled JY. Percent specific lysis ($\pm 5\text{D}$) is of JY after 4 h incubation with PBL at an E/T ratio of 50:1. IgG1 (\bullet), IgG2a (Δ), IgG2b (\square).

IgG2a ME1 did not direct lysis of Daudi cells lacking HLA-B7 (data not shown). These results demonstrate that lysis is due to ADCC. For comparing the different isotypes, an incubation time of 4 h was chosen.

Comparison of ADCC Activity Directed by Different Isotypes. ADCC directed by IgG1, IgG2b, and IgG2a ME1 antibodies at a constant E/T ratio revealed that IgG2a provides the highest level of ADCC activity, effecting a maximal specific lysis of $25 \pm 2\%$ at antibody concentrations $>2 \mu\text{g/ml}$ (Fig. 3). The IgG2b subclass is significantly less effective, directing only $10 \pm 2\%$ maximal lysis of JY. The fraction of maximal lysis directed by IgG2b and IgG2a at various concentrations, however, are the same, consistent with these antibodies having identical binding affinities for the JY target cell. In contrast, no specific lysis of JY sensitized with IgG1 ME1 was detected, even at high antibody concentrations, and even when the assay time was extended to 19 h. Furthermore, cells killed with IgG2b ME1 increased with time, but remained significantly less than that of the IgG2a-coated JY at all time points tested (Fig. 4).

We obtained similar results with the MA2.1 switch variant family (Fig. 5). IgG2a MA2.1 directed two- to threefold greater cell dependent lysis of JY at matched concentrations of IgG2b MA2.1, and IgG1 antibody gave no significant ADCC.

Quantitation of Antibody Molecules Binding JY. Radiolabeled IgG2a ME1 was titrated with JY under conditions identical to those used in the ADCC experiments. Reaching half-saturation at $1 \mu\text{g/ml}$, the binding curve closely paralleled ADCC activity. This demonstrates that a large proportion of the total HLA target molecules on JY must be bound by antibody for effective ADCC. At saturation, 5×10^6 Ig molecules bound. This approximates the number of surface HLA molecules per JY cell (33).

Inhibition of IgG2a ADCC. ADCC directed by IgG2a ME1 is inhibited equally well by IgG1 ME1 or F(ab')_2 ME1, on a molar basis (Fig. 6). This result confirms that the Fc domains of these antibodies are crucial for directing ADCC activity.

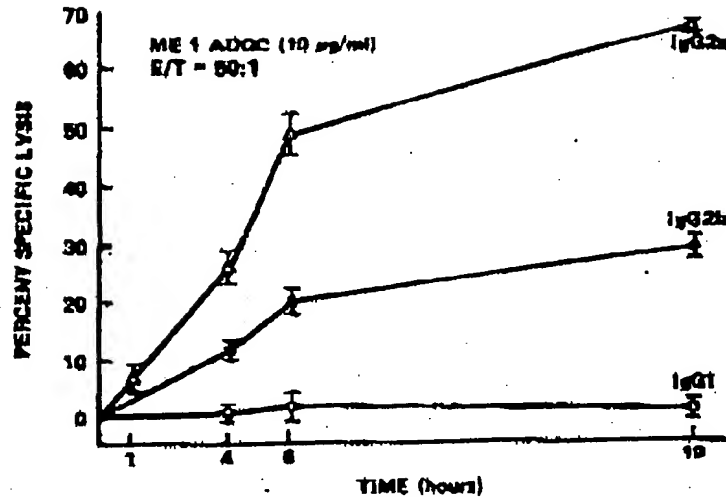


FIGURE 4. Kinetics of ADCC directed by the different isotypes of ME1. ^{51}Cr -labeled JY was incubated with antibody of each isotype at 10 $\mu\text{g}/\text{ml}$. ADCC ($\pm\text{SD}$) of PBL at an E/T ratio of 50:1 was assayed at times indicated. IgG1 (\square), IgG2a (Δ), IgG2b (\bullet).

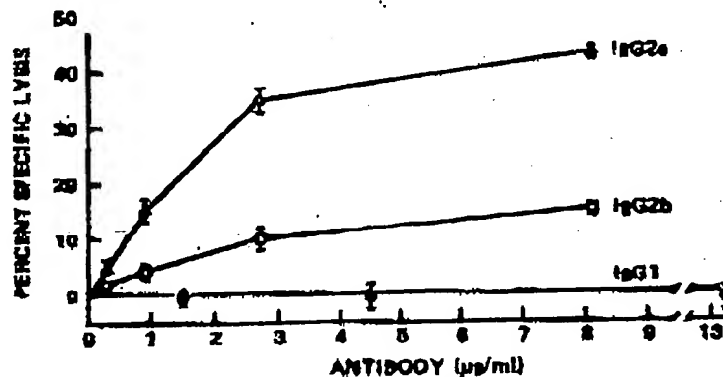


FIGURE 5. ADCC activities of different antibody isotypes of MA2.1. ^{51}Cr -labeled JY was incubated with antibody of each isotype at various concentrations. Percent specific lysis ($\pm\text{SD}$) is of JY after 4 h incubation with PBL at an E/T ratio of 50:1. IgG1 (\square), IgG2a (Δ), IgG2b (\bullet).

The Fab' of ME1 is much less effective in blocking ADCC directed by IgG2a ME1, presumably because of the lost avidity of the univalent Fab' molecules.

Comparable results for the inhibition of IgG2a-directed ADCC by IgG1 was noted in the MA2.1 system. 50% inhibition of ADCC directed by 4 $\mu\text{g}/\text{ml}$ of IgG2a MA2.1 (2.7×10^{-6} M) was achieved with an equimolar concentration of IgG1 MA2.1. IgG1 ME1, however, did not block ADCC directed by the non-cross-reacting IgG2a MA2.1. Moreover, IgG1 MA2.1 did not inhibit IgG2a ME1-directed ADCC. These results confirm that inhibition with IgG1 is due to specific competition for binding to target HLA molecules, not in the interaction with effector (K) cells.

Comparison of ADCC Activity in Different Individuals. Although there is considerable variability in the levels of ADCC activity observed with PBL isolated from different people, the relative capacities of the three Ig isotypes for directing ADCC were unchanged (Fig. 7). IgG2b ME1 directed levels of ADCC intermediate to those of IgG2a, and the IgG1 ME1 was always ineffective. Furthermore,

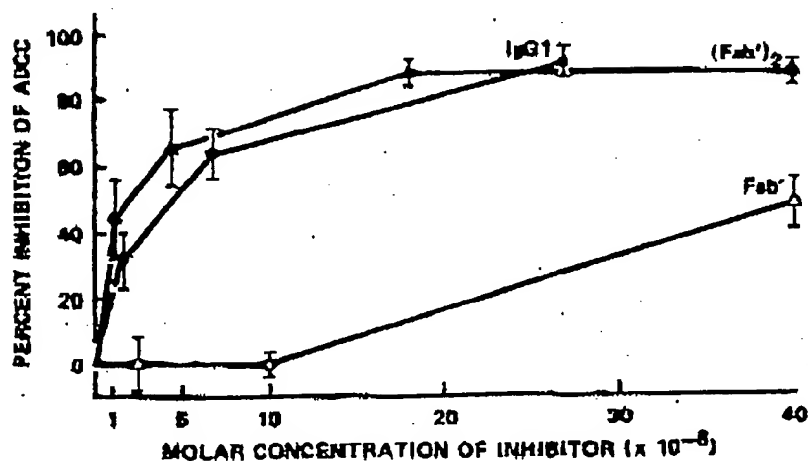


FIGURE 6. Inhibition of IgG2a ME1 ADCC. The Fab' , $F(ab')_2$, or IgG1 of ME1 was added, at various concentrations, to separate wells containing ^{51}Cr -labeled JY. IgG2a ME1 was subsequently added to 2.5 $\mu g/ml$. After a 20 min incubation at room temperature, PBL were added to an E/T ratio of 50:1. ADCC was measured after 4 h incubation at 37°C. Samples without inhibitor had 90% ($\pm 1\%$) specific lysis of JY with the IgG2a ME1. Percent inhibition of ADCC was determined by comparing the percent specific lysis of each sample with this value. IgG1 (\bullet), $F(ab')_2$ (\blacktriangle), Fab' (\triangle).

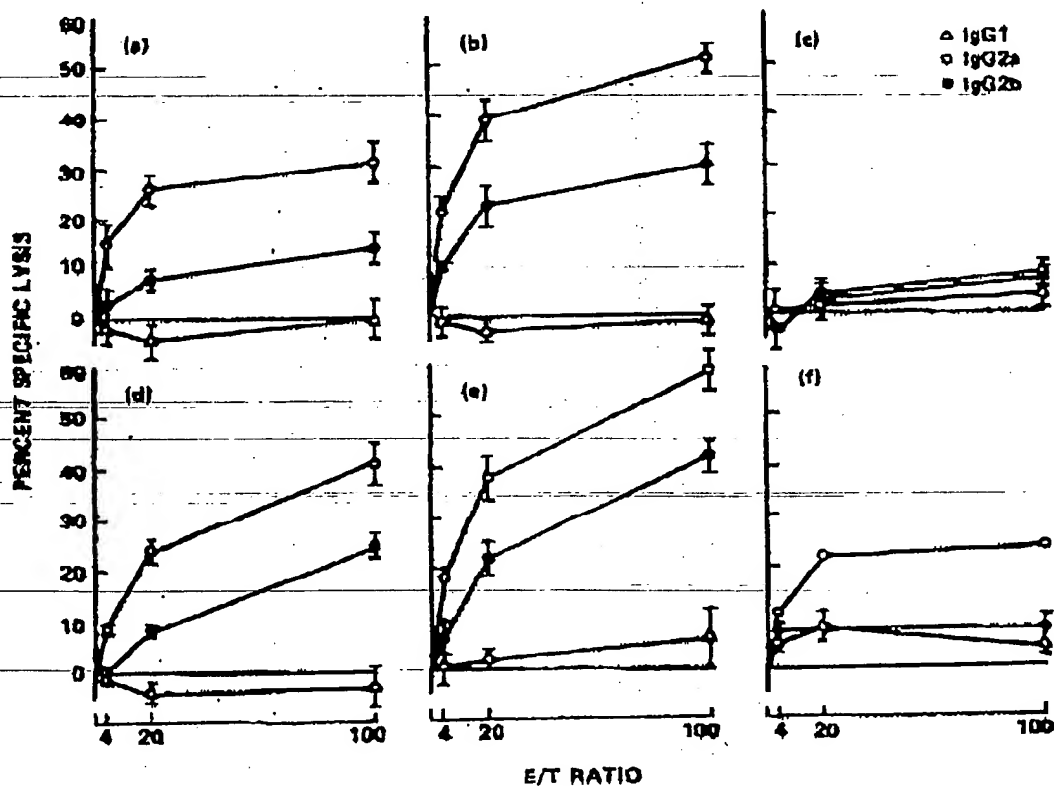


FIGURE 7. Comparison of ADCC activities of IgG1, IgG2a, and IgG2b ME1 with PBL from different individuals. Antibodies of each isotype were incubated with ^{51}Cr -labeled JY at 5 $\mu g/ml$. PBL from each individual were added at various concentrations to yield E/T ratios of 4, 20, or 100:1. Samples were assayed after 4 h incubation at 37°C. IgG1 (Δ), IgG2a (\circ), IgG2b (\bullet).

the maximum levels of ADCC with IgG2b at different E/T ratios tended to parallel the levels found with IgG2a at these E/T ratios.

Characterization of Effector Cells. To assess the ADCC activity of PBL depleted of monocytes, we compared the activity of unfractionated PBL with PBL not adhering to plastic after 2 h incubation at 37°C. These nonadherent cells represented ~85% of the freshly isolated mononuclear cells, were >98% free of monocytes (assessed by morphology after staining with Wright-Giemsa), and possessed all the ADCC activity of the total mononuclear PBL. This suggests that the PBL effector cells are nonadherent K cells (34).

Several groups of investigators (35-37) have reported the development of mAb that are specific for surface molecules expressed by human K cells. Some of these antibodies have been found to specifically block K cell ADCC activity, presumably by binding the Fc receptors of such cells (35, 37). Prior incubation of mononuclear cells with one such anti-FcR antibody, anti-Leu-11a, abrogated the ADCC activity of the PBL for IgG2a-coated JY (Table II). Other antibodies, anti-Leu-2a, anti-Leu-7, and anti-Leu-15, which reportedly bind to human K cells but do not block the FcR (38-40), gave no inhibition of ADCC directed by IgG2a anti-HLA of either specificity. Furthermore, murine IgG1 anti-DNP mAb of the same isotype as the anti-Leu-11a gave no inhibition of ADCC. Comparable results were obtained with IgG2b ME1- and IgG2a MA2.1-directed ADCC (data not shown).

Multiparameter fluorescence and light scatter analysis of PBL confirms that cells expressing Leu-11a have the forward and obtuse angle light scatter characteristics of large lymphocytes, which can readily be distinguished from cells expressing Mo2, a monocyte differentiation antigen (41). The light scatter of whole, ungated PBL is shown in Fig. 8a. A box enclosing 95% of the Leu-11a⁺ cells (Fig. 8b) excludes 96% of Mo2-expressing monocytes (Fig. 8c). All cells

TABLE II
Inhibition of ADCC by Anti-FcR Antibody

IgG2a ME1 added to JY	Antibody added to effector cells	ADCC (±SD)
+	—	29 (2)
+	Anti-Leu-2a	25 (3)
+	Anti-Leu-7	28 (2)
+	Anti-Leu-11a	2 (3)
+	Anti-Leu-15	27 (2)
+	Anti-DNP	25 (3)
—	—	0 (1)
—	Anti-Leu-2a	2 (2)
—	Anti-Leu-7	2 (1)
—	Anti-Leu-11a	0 (1)
—	Anti-Leu-15	1 (1)
—	Anti-DNP	0 (1)

Inhibition of ADCC by anti-FcR: ⁵¹Cr-labeled JY were incubated with or without IgG2a ME1 at 4 µg/ml. PBL were incubated with 1.5 µg/10⁶ cells of mAb at 25 µg/ml for 20 min at room temperature. PBL were washed free of unbound antibody and added to JY at an E/T ratio of 50:1. ADCC was assayed after 4 h incubation at 37°C.

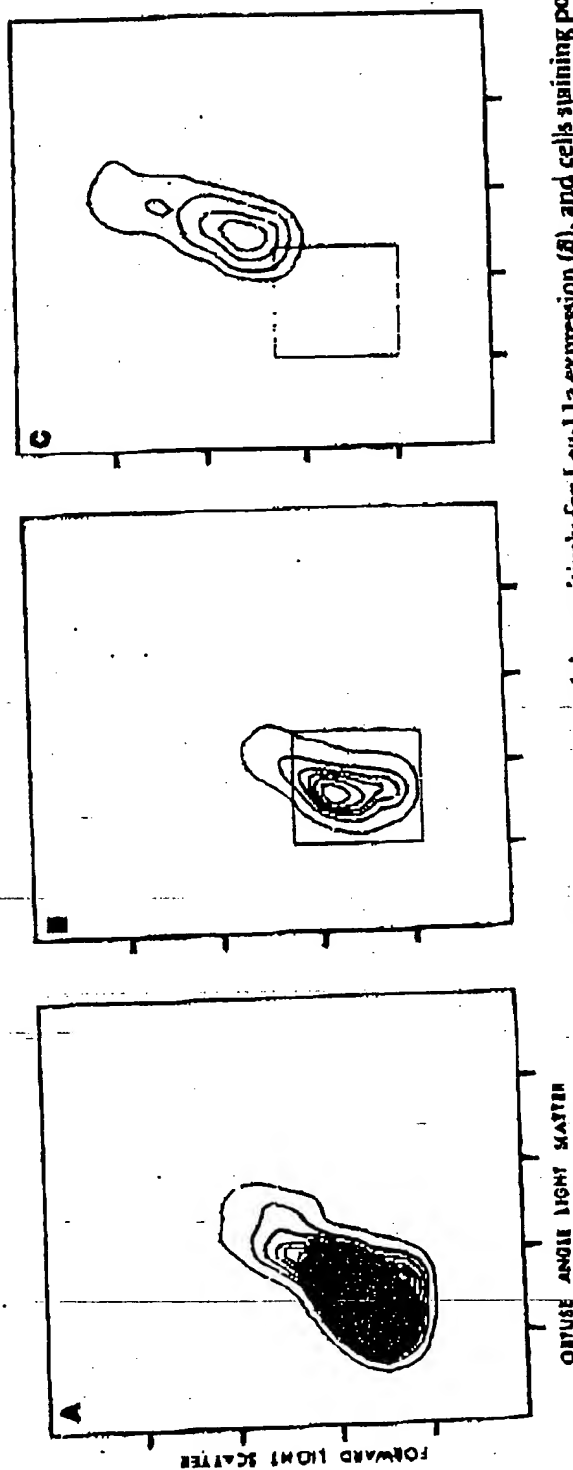


FIGURE 8. Light scatter analysis of selected cell subpopulations. PBL were stained with FITC-conjugated antibodies specific for Leu-1 Ia or Mo2. Forward angle and oblique angle light scatter of cells were measured on a modified FACS II. Light scatter contour maps are of whole, ungated PBL (A), cells staining positively for Leu-1 Ia expression (B), and cells staining positively for Mo2 (C). The box in B and C circumscribes the light scatter of 95% of the Leu-1 Ia⁺ cells.

FIGURE 11. Light scatter analysis of selected cell subpopulations. PBL were stained with FITC-conjugated antibodies specific for Leu-1 Ia or Mo2. Forward angle and oblique angle light scatter of cells were measured on a modified FACS II. Light scatter contour maps are of whole, ungated PBL (A), cells staining positively for Leu-1 Ia expression (B), and cells staining positively for Mo2 (C). The box in B and C circumscribes the light scatter of 95% of the Leu-1 Ia⁺ cells.

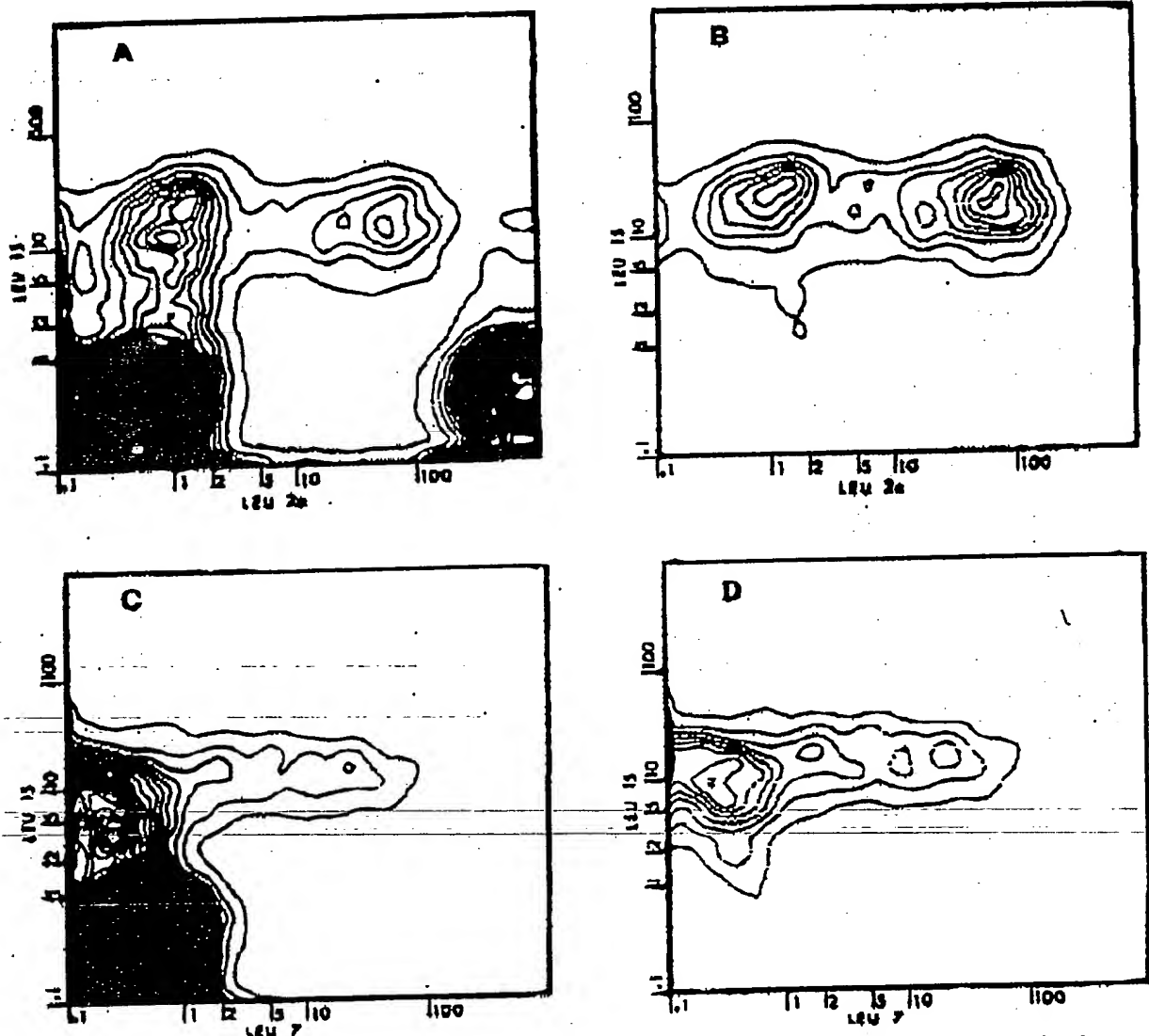


FIGURE 9. Cell surface antigen distribution of Leu-11a⁺ cells. PBL were stained with phycoerythrin-conjugated anti-Leu-15, FITC-labeled anti-Leu-11a, and biotin-conjugated anti-Leu-2a (a and b) or biotin-conjugated anti-Leu-7 (c and d). After washing away unbound antibody, cells were stained with Texas Red-avidin as a second step. a and c demonstrate the staining of ungated lymphocytes with anti-Leu-15/anti-Leu-2a, and anti-Leu-15/anti-Leu-7, respectively. b and d depict the distribution of cell surface antigens on the 10% of cells staining positively for Leu-11a. Numbers on the ordinate and abscissa indicate relative fluorescence intensity.

expressing Leu-11a also express Leu-15 (Fig. 9, b and d), the receptor for C3bi (42). Approximately half of the Leu-11a⁺ cells stained dully with anti-Leu-2a (Fig. 9b), and less than half stained positively with anti-Leu-7 (Fig. 9d).

To enrich for cells expressing Leu-11a without staining with anti-Leu-11a antibody, PBL were stained with phycoerythrin-conjugated anti-Leu-15 and sorted into separate subpopulations based on light scatter and fluorescence. PBL depleted of monocytes by FACS sorting (based on light scatter) possessed ADCC activity comparable to, or greater than, PBL from unsorted populations (Table III). Lymphocytes expressing Leu-15, however, had even greater ADCC activity.

TABLE III
IgG2a ADCC Activities of Selected Lymphoid Populations

Population	Percent of total	ADCC at various E/T ratios (\pm SD)		
		4:1	20:1	100:1
Unseparated PBL	100	6 (1)	24 (2)	52 (3)
Monocyte-depleted	95	6 (1)	31 (2)	57 (1)
Leu-15 + lymphocytes	29	15 (1)	52 (1)	NT
Leu-15 - lymphocytes	67	1 (1)	5 (3)	NT

IgG2a ADCC activities of selected lymphoid populations. Unstained PBL were sorted on the basis of light scatter to exclude monocytes. In addition, PBL were stained with phycoerythrin-anti-Leu-15 and sorted on the basis of fluorescence and light scatter. The three sorted populations were compared with the untreated PBL at various E/T ratios for ADCC activity directed by 4 μ g/ml IgG2a ME1 against 51 Cr-labeled JY. ADCC was measured after 4 h incubation at 37°C degrees. Lymphocytes were depleted of monocytes by light scatter. NT, not tested.

In contrast, cells not expressing Leu-15 had no ADCC activity, although these cells represented ~70% of the unfractionated PBL.

Discussion

In this study, we directly compared the activity of the IgG1, IgG2a, and IgG2b isotypes of otherwise identical mouse anti-HLA antibodies directing human PBL in ADCC against a B lymphoblastoid target. For antibodies of both the ME1 and MA2.1 switch variant families, we demonstrate that the IgG2a isotype is most effective, IgG2b less effective, and IgG1 completely ineffective in directing ADCC by human K cells.

The inactivity of IgG1 antibody in directing ADCC conflicts with some published reports of ADCC directed by this isotype (10, 43, 44). In contrast to the IgG1 antibodies of both ME1 and MA2.1, the IgG1 antibody that directed high levels of ADCC in one of these studies also directs efficient C-mediated cytotoxicity of antigen-bearing cells (10). Generally, of the murine IgG isotypes, only IgG2a and IgG2b are effective in C-mediated lysis (30, 45). In particular, this is true for antibodies of both ME1 and MA2.1 switch variant families (unpublished observations). Some mouse IgG1 antibodies, however, can effectively fix C (46-48). This may be due to unusual (variable-constant) V-C region interactions or posttranslational modification of the C region. This may also explain, in part, why some IgG1 can direct ADCC by human K cells. Furthermore, differences in the ADCC assay, such as higher E/T ratios, longer incubation times, or different target cells, may permit IgG1 antibodies to direct ADCC. Our studies suggest, however, that an IgG2a antibody generally would be more effective than an IgG1 antibody of the same specificity. This is in agreement with Hellstrom et al. (14) and Imai et al. (15), who noted greater effectiveness of murine IgG2a over IgG1 of like specificity in directing human PBL in ADCC against human melanoma cell lines.

Our studies differ in several important respects from those of Steplewski et al. (13), who concluded that IgG2a is the only effective murine antibody isotype for

directing lysis of a human tumor cell line by human effector cells. These investigators studied the effector activity of adherent PBL activated by weeks of *in vitro* culture. In contrast, the ADCC activities studied here are mediated by freshly isolated nonadherent Leu-15⁺ cells that are inhibited by anti-Leu-11a, an antibody that does not react with monocyte FcR.

Some of these differences may explain our finding of intermediate levels of ADCC activity directed by antibody of the IgG2b isotype, in contrast to the apparent inactivity of this subclass reported by Steplewski et al. (13). Aside from the possibility that the IgG2b antibody studied by these investigators may not have the fine binding specificity and/or affinity necessary for directing ADCC, the differences noted in the activity of IgG2b could be related to differences in the FcR of the two effector populations studied. Several mAb, including Leu-11a (37), are specific for the human K cell FcR, a pronase-sensitive, trypsin-resistant membrane protein of 52,000–58,000 mol wt (35, 47). These same mAb do not bind the human monocyte FcR, which has an apparent molecular weight of 72,000 (48). However, it has been reported recently that 3G8, an mAb that reacts with an FcR protein that co-modulates with Leu-11a on human K cells (37), also specifically binds a protein expressed by 60% of the macrophages isolated from resected lung tissue (49). This finding, plus the observation that the antigen detected by 3G8 is not expressed on monocytes unless they are cultured *in vitro* for several days, supports the idea that the FcR present on peripheral blood K cells is similar to or the same as that expressed on activated macrophages.

Our data also indicate that human K cells interact preferentially with antibody bound to cells. If free antibody readily bound to the effectors' FcR, saturation of available FcR would occur at high free antibody concentrations. This would give a prozone effect with diminished cytolysis of JY by PBL at higher than saturating concentrations of added antibody. That neither IgG2a nor IgG2b anti-HLA Ig inhibit ADCC at high antibody concentrations, as seen in Figs. 4 and 6, shows that free antibody cannot compete successfully with cell-bound antibody for FcR of human K cells. This is further supported by our inability to activate effector cells for ADCC of JY by precoating the PBL with the IgG2a ME1 or MA2.1. After washing away unbound antibody, these preincubated PBL had no cytolytic activity for uncoated JY, although they still had the expected activity against IgG2a-coated JY (data not shown).

In sharp contrast, others (14, 50–52) have demonstrated that there is a prozone effect when titrating human ADCC activity with increasing concentrations of added specific antibody. Several of these studies used antisera, not purified mAb, to direct ADCC (50–52). Titrations performed with such heterogeneous mixtures would reflect the ADCC activities directed by unknown fractions of antibodies with different isotypes. If, for example, a high affinity antibody of an isotype capable of directing ADCC, is present along with low affinity antibodies of isotypes less active in directing ADCC, ADCC would be greater at low concentrations of added antisera, where the binding of high affinity antibodies would be favored. Also, without ultracentrifugation of Ig samples before testing for ADCC (as we did in this study), preparations of mAb or antisera may contain Ig aggregates that would directly bind the FcR of effector cells. Such aggregates

would compete with antibody bound to target cells and inhibit ADCC at higher serum concentrations.

The vast differences in the capabilities of different isotypes of the ME1 and MA2.1 antibodies to direct ADCC in vitro makes us consider whether the in vivo biologic activity of a particular mAb may be dependent upon its isotype. Studies comparing the activities of different isotypes derived from switch variant families in the immunotherapy of various human diseases need to be performed to test this hypothesis.

Summary

Using the fluorescence activated cell sorter to select rare IgG2a- and IgG2b-producing variants, we developed switch variant families of hybridomas from IgG1-producing hybridomas, ME1 and MA2.1. The IgG2a and IgG2b antibodies produced by such switch variants have the same binding activities for HLA as the IgG1 antibodies produced by the parent hybridomas. Using these antibodies, we directly compared the IgG1, IgG2a, and IgG2b murine Ig isotypes for their capacities to direct human peripheral blood lymphocytes (PBL) in antibody-dependent cell-mediated cytotoxicity (ADCC) against a B lymphoblastoid cell line. We demonstrate that, for antibodies of identical binding affinity and specificity, the murine IgG2a isotype is the most effective in directing ADCC by human effector cells. The murine IgG2b directs intermediate levels of ADCC activity while IgG1 is inactive. We identified the effector cells in human PBL that mediate IgG2a or IgG2b ADCC as nonadherent-killer (K)-cells. These cells express the C3b1 receptor and have cytolytic activity which is specifically blocked by a monoclonal antibody (anti-Lcu-11a) that binds the Fc receptor (FcR) of such cells. Finally, FcR-bearing K-cells bind to target cell-bound, rather than free, IgG2a or IgG2b molecules.

We wish to thank Dr. Lewis Lanier for many helpful discussions. In addition, we thank Ms. Linda Lloyd for her help in preparing this manuscript for publication.

Received for publication 5 September 1984

References

1. Oldham, R. K. 1983. Monoclonal antibodies in cancer therapy. *J. Clin. Oncol.* 1:582.
2. Cosimi, A. B., R. C. Burton, R. B. Colvin, G. Goldstein, F. L. Delmonico, M. P. LaQuaglia, N. Tolkoff-Rubin, R. H. Rubin, J. T. Herrin, and P. S. Russell. 1981. Treatment of acute renal allograft rejection with OKT3 monoclonal antibody. *Transplantation (Baltimore)* 32:535.
3. Miller, R. A., D. G. Maloney, R. Warnke, and R. Levy. 1982. Treatment of B-cell lymphoma with antiidiotypic antibody. *New Engl. J. Med.* 306:517.
4. Miller, R. A., and R. Levy. 1981. Response of cutaneous T cell lymphoma to therapy with hybridoma monoclonal antibody. *Lancet* 1:226.
5. Dillman, R. O., D. L. Shawler, R. E. Sobol, H. A. Collins, J. C. Beauregard, S. B. Wormsley, and I. Royston. 1982. Murine monoclonal antibody therapy in two patients with chronic lymphocytic leukemia. *Blood* 59:1086.
6. Ritz, J., J. M. Pesando, S. E. Sallan, L. A. Clavell, J. Notis-McConarty, P. Rosenthal,

- and S. F. Schlossman. 1981. Serotherapy of acute lymphoblastic leukemia with monoclonal antibody. *Blood*. 58:141.
7. Miller, R. A., D. G. Maloney, J. McKillop, and R. Levy. 1981. In vivo effects of murine hybridoma monoclonal antibody in a patient with T-cell leukemia. *Blood*. 58:78.
 8. Herlyn, D., M. Herlyn, Z. Steplewski, and H. Koprowski. 1979. Monoclonal antibodies in cell-mediated cytotoxicity against human melanoma and colorectal carcinoma. *Eur. J. Immunol.* 9:657.
 9. Herlyn, D., and H. Koprowski. 1982. IgG2a monoclonal antibodies inhibit human tumor growth through interaction with effector cells. *Proc. Natl. Acad. Sci. USA*. 79:4761.
 10. Ralph, P., and I. Nakoinz. 1983. Cell-mediated lysis of tumor targets directed by murine monoclonal antibodies of IgM and all IgG isotypes. *J. Immunol.* 131:1028.
 11. Ralph, P., I. Nakoinz, B. Diamond, and D. Yelton. 1980. All classes of murine IgG antibody mediate macrophage phagocytosis and lysis of erythrocytes. *J. Immunol.* 125:1885.
 12. Steplewski, Z., D. Herlyn, G. Maul, and H. Koprowski. 1983. Hypothesis: macrophages as effector cells for human tumor destruction mediated by monoclonal antibody. *Hybridoma*. 2:1.
 13. Steplewski, Z., M. D. Lubeck, and H. Koprowski. 1983. Human macrophages armed with murine immunoglobulin G2a antibodies to tumors destroy human cancer cells. *Science (Wash. DC)*. 221:865.
 14. Hellström, I., K. E. Hellstrom, and M. Yeh. 1981. Lymphocyte-dependent antibodies to antigen 9.1, a cell-surface antigen expressed by a subgroup of human melanomas. *Int. J. Cancer*. 27:281.
 15. Imai, K., M. A. Pellegrino, B. S. Wilson, and S. Ferrone. 1982. Higher cytolytic efficiency of an IgG2a than of an IgG1 monoclonal antibody reacting with the same (or spatially close) determinant on a human high-molecular-weight melanoma-associated antigen. *Cell Immunol.* 72:239.
 16. Beyreuther, K., J. Bovers, R. Dildrop, H. Dorff, T. Geske, B. Lietegang, C. Muller, M. Neuberger, A. Radbruch, K. Rajewsky, F. Sablitzky, P. H. Schreier, and S. Zaim. 1981. Isolation and characterization of class switch variants of myeloma and hybridoma cells. ICN-UCLA Symp. Mol. Cell. Biol. 20:229.
 17. Dangi, J. L., and L. A. Herzenberg. 1982. Selection of hybridomas and hybridoma variants using the fluorescence-activated cell sorter. *J. Immunol. Methods*. 52:1.
 18. Kipps, T. J., and L. A. Herzenberg. 1984. Hybridoma immunoglobulin isotype switch variant selection using the fluorescence-activated cell sorter. In *Handbook of Experimental Immunology*. D. M. Weir, L. A. Herzenberg, C. C. Blackwell, and L. A. Herzenberg, editors. Blackwell Publications, Ltd., Edinburgh. Fourth ed. In press.
 19. Terhorst, C., R. Robb, C. Jones, and J. L. Strominger. 1977. Further structure studies of the heavy-chain of HLA antigens and its similarity to immunoglobulins. *Proc. Natl. Acad. Sci. USA*. 74:4002.
 20. Ellis, S. A., C. Taylor, and A. McMichael. 1982. Recognition of HLA-B27 and related antigen by a monoclonal antibody. *Hum. Immunol.* 5:49.
 21. McMichael, A. J., P. Parham, N. Rust, and F. M. Brodsky. 1980. A monoclonal antibody that recognizes an antigenic determinant shared by HLA-A2 and HLA-B17. *Hum. Immunol.* 1:121.
 22. Huang, C., M. Parson, V. T. Oi, H. S. Huang, and L. A. Herzenberg. 1983. Genetic characterization of mouse immunoglobulin allotypic determinants (allotopes) defined by monoclonal antibodies. *Immunogenetics*. 18:311.

23. Goding, J. W. 1976. Conjugation of antibodies with fluorochromes: modification to the standard methods. *J. Immunol. Methods*. 13:215.
24. Parks, D. R., L. L. Lanier, and L. A. Herzenberg. 1984. Flow cytometry and fluorescence-activated cell sorting. In *Handbook of Experimental Immunology*. D. M. Weir, L. A. Herzenberg, C. C. Blackwell, and L. A. Herzenberg, editors. Blackwell Publications, Ltd., Edinburgh. Fourth ed. In press.
25. Lingren, B. W. 1968. Statistical Theory. Macmillan Co., New York, N.Y.
26. Hunter, W. H., and F. C. Greenwood. 1962. Preparation of iodine-131-labeled human growth hormone of high specific activity. *Nature (Lond.)*. 194:495.
27. Tsu, T. T., and L. A. Herzenberg. 1980. Solid-phase radioimmune assays. In *Selected Methods in Cellular Immunology*. B. B. Mishell, and S. M. Shügi, editors. W. H. Freeman and Co., San Francisco. 373-397.
28. Ey, P. L., S. J. Prowse, and C. R. Jenkin. 1978. Isolation of pure IgG1, IgG2a, and IgG2b immunoglobulins from mouse serum using protein A-Sepharose. *Immunochemistry*. 15:429.
29. Parham, P. 1984. Preparation and purification of active fragments from mouse monoclonal antibodies. In *Handbook of Experimental Immunology*. D. M. Weir, L. A. Herzenberg, C. C. Blackwell, and L. A. Herzenberg, editors. Blackwell Publications, Ltd., Edinburgh. Fourth ed. In press.
30. Parham, P., T. J. Kippa, F. E. Ward, and L. A. Herzenberg. 1983. Isolation of heavy chain class-switch variants of a monoclonal anti-DC1 hybridoma cell line: effective conversion of non-cytotoxic IgG1 antibodies to cytotoxic IgG2 antibodies. *Hum. Immunol.* 8:141.
31. Kippa, T. J., and L. A. Herzenberg. 1984. The fluorescence-activated cell sorter: complementary tools in immunodiagnosis and immunotherapy. In *Rapid Methods in Immunology and Microbiology*. Springer-Verlag, Berlin. In press.
32. Ways, J. P., and P. Parham. 1983. The binding of monoclonal antibodies to cell-surface molecules. *Biochem. J.* 216:423.
33. Parham, P., C. J. Barnstable, and W. F. Bodmer. 1979. Use of a monoclonal antibody (W6/32) in structural studies of HLA-A, B, C antigens. *J. Immunol.* 123:343.
34. Perlmann, P., H. Perlmann, and H. Wigzell. 1972. Lymphocyte-mediated cytotoxicity in vitro. Induction and inhibition by humoral antibody and nature of effector cells. *Transplant. Rev.* 13:91.
35. Perussia, B., O. Acuto, C. Terhorst, J. Faust, R. Lazarus, V. Fanning, and G. Trinchieri. 1983. Human natural killer cells analyzed by B73.1, a monoclonal antibody blocking Fc receptor functions. II. Studies of B73.1 antibody-antigen interaction on the lymphocyte membrane. *J. Immunol.* 130:2142.
36. Phillips, J. H., and G. F. Babcock. 1983. NKP-15: a monoclonal antibody reactive against purified human natural killer cells and granulocytes. *Immunol. Lett.* 6:143.
37. Perussia, B., G. Trinchieri, A. Jackson, N. L. Warner, J. Faust, H. Rumpold, D. Kraft, and L. L. Lanier. 1984. The Fc receptor of IgG on human natural-killer cells: phenotypic, functional, and comparative studies using monoclonal antibodies. *J. Immunol.* 133:180.
38. Abo, T., and C. M. Balch. 1981. A differentiation antigen of human NK and K cells identified by a monoclonal antibody (HNK-1). *J. Immunol.* 127:1024.
39. Perussia, B., V. Fanning, and G. Trinchieri. 1983. A human NK and K cell subset shares with cytotoxic T cells expression of the antigen recognized by antibody OKT8. *J. Immunol.* 131:223.
40. Lanier, L. L., A.-M. Le, J. H. Phillips, N. L. Warner, and G. F. Babcock. 1983. Subpopulations of human natural killer cells defined by expression of the Leu-7 (HNK-1) and Leu-11 (NK-15) antigens. *J. Immunol.* 131:1789.

41. Todd, R. F., III, L. M. Nadler, and S. F. Schlossman. 1981. Antigens on human monocytes identified by monoclonal antibodies. *J. Immunol.* 126:1435.
42. Beller, D. I., T. A. Springer, and R. D. Schreiber. 1982. Anti-Mac-1 selectively inhibits the mouse and human type three complement receptor. *J. Exp. Med.* 156:1000.
43. Imai, K., A.-K. Ng, and S. Ferrone. 1981. Characterization of monoclonal antibodies to human melanoma-associated antigens. *J. Natl. Cancer Inst.* 66:489.
44. Greenberg, A. H., and P. M. Lydyard. 1979. Observations of IgG1 anti-DNP hybridoma-mediated ADCC and the failure of three IgM anti-DNP hybridomas to mediate ADCC. *J. Immunol.* 123:861.
45. Ey, P. L., G. J. Russell-Jones, and C. R. Jenkin. 1980. Isotypes of mouse IgG-I. Evidence for 'non-complement-fixing' IgG1 antibodies and characterization of their capacity to interfere with IgG2 sensitization of target red blood cells for lysis by complement. *Mol. Immunol.* 17:699.
46. Ey, P. L., S. J. Prowse, and C. R. Jenkin. 1979. Complement-fixing IgG1 constitutes a new subclass of mouse IgG. *Nature (Lond.)* 281:492.
47. Neuberger, M. S., and K. Rajewsky. 1981. Activation of mouse complement by monoclonal mouse antibodies. *Eur. J. Immunol.* 11:1012.
48. Hirayama, N., T. Hirano, G. Kohler, A. Kurata, K. Okumura, and Z. Ovary. 1982. Biological activities of antiirinitrophenyl and antidinitrophenyl mouse monoclonal antibodies. *Proc. Natl. Acad. Sci. USA.* 79:613.
49. Perussia, B., G. Trinchieri, and J. Cerottini. 1979. Functional studies of Fc receptor-bearing human lymphocytes: effect of treatment with proteolytic enzymes. *J. Immunol.* 123:681.
50. Anderson, C. L. 1982. Isolation of the receptor for IgG from a human monocyte cell line (U937) and from human peripheral blood monocytes. *J. Exp. Med.* 156:1794.
51. Springer, T. A., and J. C. Unkeles. 1984. Analysis of macrophage differentiation and function with monoclonal antibodies. In *Macrophage Activation*. D. O. Adams and M. G. Hanna, editors. Plenum Publishing Co., New York. 1-12.
52. Shen, L., and M. W. Fanger. 1981. Secretory IgA antibodies synergize with IgG in promoting ADCC by human polymorphonuclear cells, monocytes, and lymphocytes. *Cell. Immunol.* 59:75.
53. Perlmann, H., P. Perlmann, L. Moretta, and M. Ronnholm. 1981. Regulation of IgG antibody-dependent cellular cytotoxicity in vitro by IgM antibodies. Mechanism and characterization of effector lymphocytes. *Scand. J. Immunol.* 14:47.
54. McCarley, D. L., V. O. Shah, and R. S. Weiner. 1983. Purified human monocyte subsets as effector cells in antibody-dependent cellular cytotoxicity (ADCC). *J. Immunol.* 131:1780.

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☒ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☒ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.